Improving HRPF Reproducibility through End-to-End Automation: Adalimumab Case Study Comparing Manual and AccelerOme-Automated Digestion with and without TMT Multiplexing

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Structural Biology the Easy Way

Computational biology and artificial intelligence (AI) are revolutionizing therapeutic development by rapidly generating structural and interaction models for biotherapeutics and small molecules. Despite significant investments and advances in AI, empirical validation remains crucial due to frequent predictive failures in dynamic protein conformations, allosteric changes, and intrinsically disordered regions. Conventional validation techniques (NMR, X-ray crystallography, cryo-EM) are costly, slow, and require extensive sample quantities, hindering timely therapeutic advancements.

GenNext's AutoFox Protein Footprinting System overcomes these challenges, providing rapid empirical validation and high-resolution insights into protein structure and interactions at significantly lower costs and sample requirements.

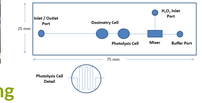


Figure 1: *HRPF labeling is performed in an automated fashion using a 96-well plate in the AutoFox System. Sample delivery, reagent mixing, and flashing are performed through a microfluidic chip designed to maximize irradiated volume. Samples are quenched in an adjacent well prior to LC-MS/MS analysis.*

Fully Automated HRPF Labeling

The AutoFox System uses a proprietary flash oxidation lamp to generate hydroxyl radicals (•OH), rapidly modifying solvent-exposed amino acid side chains. These covalent modifications provide a direct, quantitative measure of solvent accessibility and conformational dynamics, offering critical insights into protein higher order structure. With fully automated and reproducible labeling, the AutoFox enables precise spatial mapping of protein folding, surface topology, and interaction interfaces—supporting high-confidence analysis of protein–protein and protein–ligand interactions.

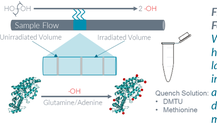


Figure 2: Schematic of the AutoFox Footprinting method.
With this method, protein is mixed with hydrogen peroxide and flowed passed a flash lamp which photolyzes the hydrogen peroxide into two •OH and modifies solvent exposed amino acids. Following labeling, the sample is deposited into a quench solution of DMTU and methionine.

Protein Footprinting with the AutoFox System

Precision. Reproducibility. Confidence.

The AutoFox System delivers highly reproducible and accurate hydroxyl radical protein footprinting (HRPF) data—empowering confident decision-making in biopharmaceutical discovery and development.

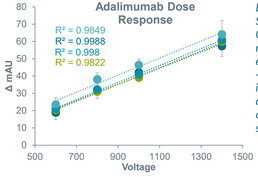


Figure 3: High Reproducibility of Protein Dose Response Curves Using the AutoFox System Dose response curves generated by the AutoFox System exhibit strong linear correlation ($R^2 > 0.98$) between hydroxyl radical concentration (Δ mAU) and the voltages applied (V) with excellent relative standard deviations (RSDs) of ~1–11% across three technical replicates. Four independent biological replicates were conducted on different days, using separate chips and operators, demonstrating the system's robust day-to-day reproducibility.

HRPF Workflow with Automated Digestion and TMT Multiplexing

HRPF was performed on adalimumab using the AutoFox® System. Following HRPF, samples were divided and either digested manually using a standard bottom-up proteomics workflow or digested automatically with sample preparation kits on the Thermo Scientific™ AccelerOme™ automated sample preparation platform. The automated digestion products were analyzed either label-free or with TMT multiplexing for improved quantification. All digested samples were subsequently analyzed by LC-MS/MS using Thermo Scientific™ Vanquish™ Neo UHPLC system and Thermo Scientific™ Orbitrap™ Ascend Structural Biology Tribrid™ mass spectrometer, and data were processed using both FoxWare® Software and Thermo Scientific™ Proteome Discoverer™ software.

Automated Digestion and TMT Labeling Streamline HRPF Sample Preparation and Analysis

Automating the digestion step with the AccelerOme automated sample preparation platform reduced hands-on time and shortened the digestion process from overnight to just one hour. Incorporating TMT multiplexing further reduced MS run time by consolidating replicates into a single analysis.

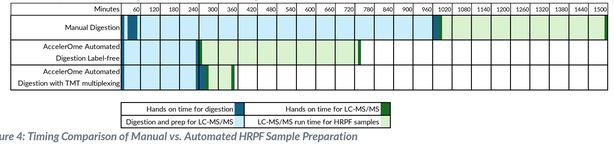


Figure 4: Timing Comparison of Manual vs. Automated HRPF Sample Preparation
HRPF samples of adalimumab (3 controls, 5 labeled) were prepared using either a manual bottom-up digestion workflow or an automated digestion protocol on the AccelerOme automated sample preparation platform. Manual preparation required overnight trypsin digestion (light blue, passive time) with significant hands-on setup (dark blue). In contrast, the automated workflow reduced the trypsin digestion to one hour, greatly minimized hands-on effort and passive incubation times. Following digestion, samples were analyzed by LC-MS/MS either individually (label-free) or multiplexed with TMT labeling. LC-MS/MS analysis is represented by dark green (hands-on instrument setup) and light green (instrument runtime). Multiplexing with TMT substantially reduced total LC-MS/MS runtime by consolidating replicates into a single run, thereby decreasing instrument burden and improving throughput. This time saving will further increase with the complexity of the HRPF experiment and higher TMT-plex.

Automated Digestion Maintains Peptide Oxidation with Improved Reproducibility

Peptide oxidation levels are comparable between manual and automated label-free digestion workflows, demonstrating that automation preserves the integrity of HRPF readouts. Importantly, the automated workflow provided modest improvements in reproducibility, as evidenced by lower relative standard deviations across replicate analyses. This establishes automated digestion as a reliable alternative to manual workflows while reducing variability in peptide-level quantification.

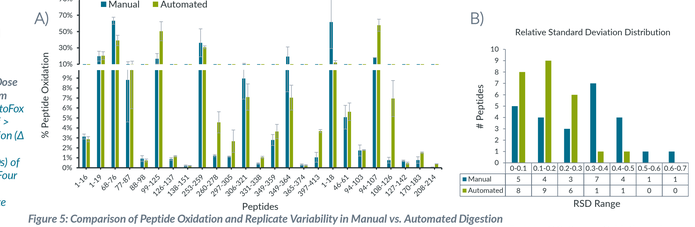


Figure 5: Comparison of Peptide Oxidation and Replicate Variability in Manual vs. Automated Digestion
(A) Histogram of peptide oxidation levels for manual (blue) and automated (green) digestion, calculated in FoxWare®Software. Oxidation values were determined from chromatographic peak areas of modified peptide extracted ion chromatograms relative to the total peptide signal. The results demonstrate similar peptide oxidation patterns across workflows. (B) Distribution of relative standard deviations (RSDs) for peptides processed by manual versus automated digestion. The automated workflow showed a shift toward lower RSD values, indicating improved reproducibility of peptide oxidation quantification.

Sequence Coverage of Oxidized Peptides Across Workflows

Manual and automated digestion achieved 90% sequence coverage, compared to 73% with TMT multiplexing. The slightly lower coverage in TMT workflow is likely due to an additional peptide clean-up step not performed in the other methods. Despite this, coverage remained sufficient for quantitative oxidation analysis, and automated digestion was better suited for identifying specific oxidized residues (highlighted in orange). Overall, automated workflows, with or without TMT labeling, preserved structural insights while delivering increased throughput and reproducibility.

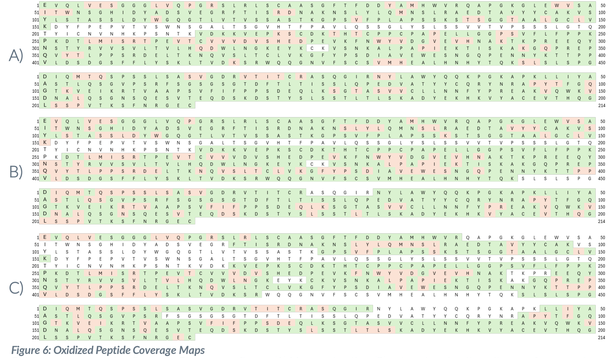


Figure 6: Oxidized Peptide Coverage Maps
Data were analyzed using Proteome Discoverer software. (A) Manual digestion, (B) automated digestion with label-free analysis, and (C) automated digestion with TMT multiplexing. Peptides detected are highlighted in green, with specific oxidized residues highlighted in orange.

Automated Digestion Improves Sample Quality and Reproducibility

Automated digestion reduced missed cleavages compared to manual preparation, resulting in cleaner peptide datasets. The number of identified modifications increased with automation, reflecting more complete peptide characterization. Replicate reproducibility was also improved, with the greatest gains observed when TMT multiplexing was applied, highlighting the combined benefit of automation and labeling for robust HRPF analysis.

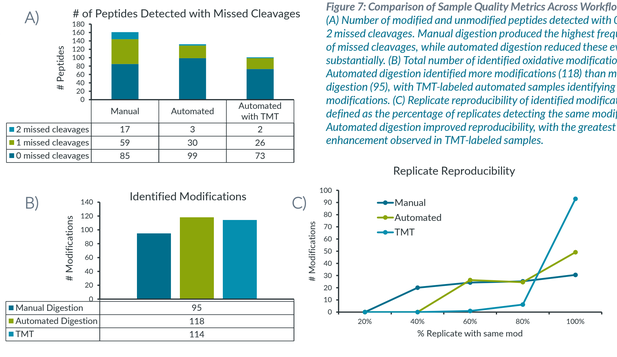


Figure 7: Comparison of Sample Quality Metrics Across Workflows
(A) Number of modified and unmodified peptides detected with 0, 1, or 2 missed cleavages. Manual digestion produced the highest frequency of missed cleavages, while automated digestion reduced these events substantially. (B) Total number of identified oxidative modifications. Automated digestion identified more modifications (118) than manual digestion (95), with TMT-labeled automated samples identifying 114 modifications. (C) Replicate reproducibility of identified modifications, defined as the percentage of replicates detecting the same modification. Automated digestion improved reproducibility, with the greatest enhancement observed in TMT-labeled samples.

TMT Multiplexing Significantly Improves Replicate Reproducibility

Residue-level modification reproducibility, measured by relative standard deviation (RSD), was comparable between manual and automated label-free digestion workflows. In contrast, automated digestion with TMT multiplexing provided a marked improvement, with most modifications showing low RSD values and a substantially reduced average RSD. This demonstrates that TMT labeling not only consolidates replicate analyses but also enhances the reliability of HRPF quantification, strengthening confidence in structural interpretation for biopharma applications.

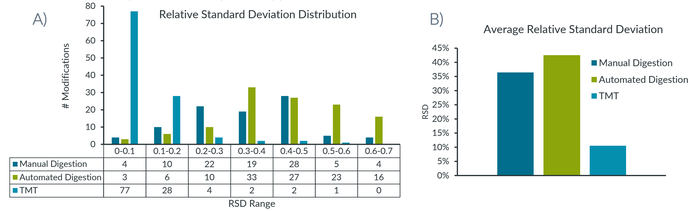
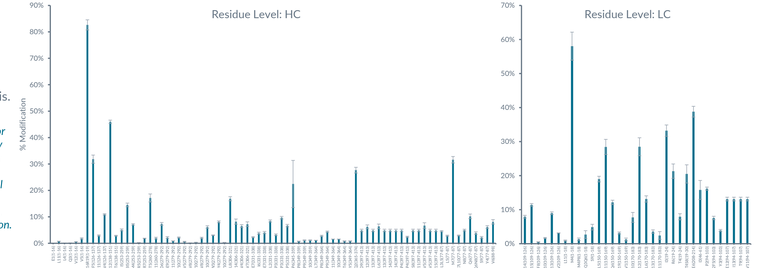


Figure 8: Relative Standard Deviation of Residue-Level Modifications Across Workflows
(A) Distribution of RSD values for modifications detected in manual digestion, automated label-free digestion, and automated TMT multiplexed samples. Manual and automated digestion showed similar distributions, while TMT labeling shifted most modifications into lower RSD ranges.(B) Average RSD values across workflows, demonstrating the significant reduction in variability achieved with TMT labeling compared to manual and automated digestion alone.

Residue-Level Oxidation Results from TMT Multiplexed Samples

Residue-level HRPF analysis of adalimumab TMT samples revealed detailed oxidation patterns across both the heavy chain (HC) and light chain (LC). Oxidized residues were detected at multiple sites with varying extents of modification, demonstrating that automated digestion with TMT preserves site-specific resolution of HRPF readouts. These results confirm that the TMT-enabled workflow provides robust residue-level structural information while improving reproducibility and throughput, making it well-suited for biopharma applications requiring precise mapping of protein higher-order structure.



Conclusions

- Combining the AutoFox® System with the AccelerOme automated sample preparation platform and TMT multiplexing delivers a fully automated, end-to-end HRPF workflow.
- Automation significantly reduces hands-on effort and shortens digestion from overnight to one hour, while TMT multiplexing decreases MS instrument time by consolidating replicates.
- Automated digestion reduces missed cleavages, increases the number of identified HRPF modifications, and improves replicate reproducibility.
- TMT labeling further enhances reproducibility by lowering relative standard deviation, enabling more confident quantification of HRPF modifications.
- Together, these advances establish an efficient, reproducible, and scalable HRPF workflow, providing higher confidence in structural insights. This directly supports biopharma applications by accelerating the study of protein higher-order structure and improving characterization of complex biotherapeutics. PO004272-EN

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